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Validation of leaf enzymes in the detergent and textile industries: launching of a new platform technology

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Summary

Chemical catalysts are being replaced by biocatalysts in almost all industrial applications due to environmental concerns, thereby increasing their demand. Enzymes used in current industries are produced in microbial systems or plant seeds. We report here five newly launched leaf-enzyme products and their validation with 15 commercial microbial-enzyme products, for detergent or textile industries. Enzymes expressed in chloroplasts are functional at broad pH/temperature ranges as crude-leaf extracts, while most purified commercial enzymes showed significant loss at alkaline pH or higher temperature, required for broad range commercial applications. In contrast to commercial liquid enzymes requiring cold storage/transportation, chloroplast enzymes as a leaf powder can be stored up to 16 months at ambient temperature without loss of enzyme activity. Chloroplast-derived enzymes are stable in crude-leaf extracts without addition of protease inhibitors. Leaf lipase/mannanase crude extracts removed chocolate or mustard oil stains effectively at both low and high temperatures. Moreover, leaf lipase or mannanase crude-extracts removed stain more efficiently at 70 °C than commercial microbial enzymes (<10% activity). Endoglucanase and exoglucanase in crude leaf extracts removed dye efficiently from denim surface and depilled knitted fabric by removal of horizontal fibre strands. Due to an increased demand for enzymes in the food industry, marker-free lettuce plants expressing lipase or cellobiohydrolase were created for the first time and site-specific transgene integration/homoplasmy was confirmed by Southern blots. Thus, leaf-production platform offers a novel low-cost approach by the elimination of fermentation, purification, concentration, formulation and cold-chain storage/transportation. This is the first report of commercially launched protein products made in leaves and validated with current commercial products.

Introduction

Several advantages of the biocatalyst (enzyme) over chemical catalysts including their environment-friendly nature make them a favourable choice for almost all industrial applications. As a result, their demand is steadily increasing. The detergent industry is among the top consumers of enzymes. Proteases, lipases and amylases are used as additives in detergent formulations. Lipases are used in the detergent industry to decompose fatty materials that are major components of stain produced by oils or butter. Lipases play a pivotal role in several different commercial applications. Lipases dominate the enzyme market and account for 70% of enzyme sales, along with proteases (Li *et al.*, 2012). Most of the lipases are stable in organic solvents, do not require a cofactor for activity and possess a broad substrate specificity making them suitable for diverse commercial applications (Jaeger and Manfred, 1998). However, current commercial lipases have some limitations because of their poor performance/stability in alkaline pH or higher temperature (detergents are alkaline and washing machines use hot water).

Another important class of enzymes that are used in laundry detergents is mannanases. Mannanases hydrolyse β-1-4 linkages in the mannan backbone. Mannans are most commonly found in chocolate, tomato ketchup, ice cream and personal care products as thickening agents or stabilizers, which are unfortunately responsible for most cloth stains (Bettiol *et al.*, 2000; Chauhan *et al.*, 2012). Mannan-containing stains are difficult to remove because they adsorb to the cellulose fibres of cotton fabrics by hydrogen bonding. The ability of mannanase to hydrolyse insoluble mannan into a smaller polymer of mannose (oligosaccharide) makes them water soluble (Dhawan and Kaur, 2007) that flushes off stain at the time of fabric washing or rinsing. This makes mannanase valuable in laundry or dishwashing detergents. However, mannanase products that maintain their activity through a wide range of temperature and in the presence of surfactants are important unmet need of industries (Sarmiento *et al.*, 2015). Other commercial applications for mannanase enzyme include the paper and pulp industries, bioethanol production, oil and gas well stimulation, food and feed, coffee extraction, nutraceuticals and pharmaceuticals (Srivastava and Kapoor, 2017; Van Zyl *et al.*, 2010).

Cellulases are a group of endoglucanases and exoglucanase enzymes, used in various industrial applications as components of detergents, animal feed additives and as biocatalysts for textile processing (Ben and Gargouri, 2017). Cotton fabric is made of cellulose, and its breakdown requires synergistic action on the β-1-4-glycosidic bonds by endoglucanases, exoglucanases and β-D-glucosidases (Ben and Gargouri, 2017). The demand for these enzymes has exponentially increased as industrial use of toxic chemicals decreased and coincidentally minimized adverse effects on fabric texture and fibre strength (Sharma *et al.*, 2017). Endoglucanases and exoglucanases are used in detergents to enhance cleaning, colour brightness and fabric softening (Agrawal, 2017). In biowashing, cellulases remove dye from the

fibril surface with minimal damage to fabrics (Araujo *et al.*, 2008). Food processing industries use a combination of cellulases, hemicellulases and pectinases for fruit juice clarification, puree concentration and viscosity reduction (Brito and Vaillant, 2012; Sharma *et al.*, 2014). Cellulases are also extensively used in the textile industry for denim biostoning, biowashing and biopolishing (Agrawal, 2017; Anish *et al.*, 2007; Miettinen-Oinonen and Suominen, 2002).

Commercial applications of enzymes require low-cost production in large quantities. Current industries entirely depend on microbial production platform for enzyme production. Unfortunately, the cost of current microbial enzymes is prohibitive and limits their extensive use in various industrial/biological applications. Decade-old microbial production systems require prohibitively expensive fermentation facilities, purification from host cells, formulation to increase concentration, stabilizing agents and cold storage/transportation. Moreover, scalability of microbial product has always been a challenge. Therefore, there is a great need to explore novel production platform technologies that could eliminate these prohibitively expensive enzyme processes.

Easy scalability and low-cost production (Ma *et al.*, 2003) are two important factors that make plant production of enzymes a suitable alternative for microbial production. Almost all genetically modified (GM) plant products are derived from seeds. Although endoglucanases (Gray *et al.*, 2009; Harrison *et al.*, 2011), exoglucanase (Harrison *et al.*, 2011, 2014), mannanase (Agrawal *et al.*, 2011; Hoshikawa *et al.*, 2012) and lipase (Gruber *et al.*, 2001; Lakshmi *et al.*, 2013; Pereira *et al.*, 2013) have been expressed in leaves, no leaf-based protein/enzyme commercial product has been launched so far. Therefore, in this study, we report production of lipase, mannanase, endoglucanase and exoglucanase in tobacco or lettuce chloroplasts, characterization of enzyme activity at different pH and temperature in crude leaf extracts without need for purification, stability over long-term storage of dried plant cells at ambient temperature and efficacy validation of stain removal, biowashing and biopolishing, when compared with current microbial products in detergent or textile industries. This is the first report of commercial leaf-enzyme products.

Results

Temperature and pH optima of crude leaf extracts and commercial enzyme products

The enzymes (Cp-Eg1, Cp-CelD, Cp-lipase, Cp-mannanase) expressed in tobacco chloroplasts (Cp) were evaluated in crude leaf extracts and compared with microbial commercial products for temperature and pH optima in three independent biological samples. Commercial enzymes were not chosen based on any specific criteria, and all 19 enzymes that could be obtained from different sources were evaluated (Table 1). Ten commercial products were in liquid form, and nine were in powder form (PhylloZyme products, Lipase-10, Bioprime LDNS 8511) or granulated (Novoprime A 868, Mannaway, Alkaline lipase). As per manufacturer's instructions, all commercial products were stored at 4 °C except PhylloZyme leaf-enzyme products, and Novoprime A 868 and Alkaline lipase were stored at ambient temperature. For PhylloZyme products (Cp-Eg1, Cp-CelD, Cp-lipase, Cp-mannanase), transplastomic plants expressing enzymes were grown in the greenhouse and Fraunhofer hydroponic production system, harvested and lyophilized. Lyophilized leaf materials were ground three times at full speed (pulse in 10 and out of 30 s) in a coffee mill. The fine powder used in investigations was stored with silica gel in containers at ambient temperature for 10–12 months. Enzymes were expressed in different tobacco commercial cultivars: Cp-CelD Petit Havana or TN90; Cp-lipase in LAMD or TN90; and Cp-Eg1 LAMD. LAMD is a low nicotine cultivar. Evaluation was based on enzyme equivalency and not based on weight or protein concentration because product packages did not report source or origin of enzyme, units, concentration or details of formulation (non-enzyme products or stabilizing agents or filler materials).

Endoglucanases (CelD) from *Clostridium thermocellum* and Eg1 from *Trichoderma reesei* expressed in tobacco chloroplasts were compared with ten commercial endoglucanases in three independent biological samples (Table 1) in broad pH (2–12) (Figure 1a,b) and temperature (30–90 °C) range (Figure 2a,b). All commercial endoglucanases showed the highest (100%) activity at pH 5.0, and Bioprime LDNS 8511 showed 100% activity at pH 6. All chloroplast endoglucanases (Cp-CelD, Cp-Eg1) in different cultivars showed 100% activity at pH 7. In addition, both Cp-CelD PH and Cp-CelD TN90 showed broad pH (5–9) optima with ≥90% activity. In sharp contrast, Cp-Eg1 showed 48% loss of activity at pH 10, confirming that it is not the expression in chloroplasts but the origin of an enzyme (gene) plays a significant role in determining enzyme characteristics. Most commercial enzymes including AC-100 (Jiangsu Boli Bioproducts, Taizhou, Jiangsu, China), Acid Cellulase (Sinobios, Shanghai, China), Cellulase ACx 8000L/8000P (Enzyme Supplies, Oxford, UK), and Cellulase G-CL (Enzyme Supplies) lost 85%–90% of activity at pH 10. Bioprime LDNS 8511® (Biogreen, Bangalore, Karnataka, India), Cellulase NC-100 (Jiangsu Boli Bioproducts) and Novoprime A 868® (Novozymes, Franklinton, NC) maintained > 50% activity at pH 10.

Endoglucanases showed different temperature optima. Cp-CelD, ACx 8000L/8000P, Cellulase AC-100 and Acid Cellulase showed 100% activity at 60 °C. Cp-Eg1, LDNS 8511, LX 1002, Cellusoft and Cellulase G-CL showed 100% activity at 50 °C. Novoprime A 868, Neutral Cellulase and NC-100 showed the lowest temperature optima, with 100% at 40 °C. Almost all enzymes showed decline in activity at higher temperatures. Cp-CelD, Acx 8000L/8000P and Acid Cellulase are the best performing enzymes at 60 °C.

Cp-mannanase crude leaf extract from ground leaf powder stored for 10–12 months was compared with commercial microbial enzyme Mannaway® (Novozymes) in three independent biological samples for temperature optima (Table 1). Mannaway is one of the most prominent commercial mannanases used in laundry detergents. Cp-mannanase crude extract showed maximum activity at 70 °C while commercial Mannaway showed maximum activity at 50 °C (Figure 2d). Cp-lipase crude leaf extract was compared with four commercial microbial lipases, that is LP-100L®, LP-10® (Jiangsu Boli Bioproducts), Alkaline Lipase® (Creative Enzymes, Shirley, NY) and Lipase NL-GX® (Enzyme Supplies) for temperature optima. Cp-lipase crude extract showed maximum activity at 70 °C while all tested commercial lipases LP-10, LP-100L, Alkaline Lipase and Lipase NL-GX showed maximum activity at 30 °C (Figure 2c). Moreover, Cp-lipase showed >60% activity at broad temperature range (30–80 °C), while commercial lipases showed <10% activity at 70–80 °C. Most washing machines use hot water at 60–70 °C, making commercial lipases less efficient.

Table 1 Commercial products used in this study, their manufacturer/supplier, predicted origin of transgene expressed, and storage format and temperature requirements

Sample	Company	Organism	Powder/liquid	Storage
Endoglucanases				
Cp-Eg1	PhylloZyme	*Trichoderma reesei*	Powder	Ambient
Cp-CelD	PhylloZyme	*Clostridium thermocellum*	Powder	Ambient
Bioprime LX-1002	Biogreen	*Trichoderma reesei?*	Liquid	4 °C
Bioprime LDNS8511	Biogreen	*Trichoderma reesei?*	Powder	4 °C
Cellulase AC-100	Jiangsu Boli Bioproducts	*Trichoderma reesei*	Liquid	4 °C
Cellulase NC-100	Jiangsu Boli Bioproducts	*Trichoderma reesei*	Liquid	4 °C
Neutral Cellulase	Sinobios	*Trichoderma reesei?*	Liquid	4 °C
Acid Cellulase	Sinobios	*Trichoderma reesei?*	Liquid	4 °C
Cellulase ACx	Enzyme Supplies	*Trichoderma reesei*	Liquid	4 °C
Cellulase G-CL	Enzyme Supplies	*Trichoderma reesei?*	Liquid	4 °C
Cellusoft L	Novozymes	*Trichoderma reesei*	Liquid	4 °C
Novoprime A 868	Novozymes	*Humicola insolens*	Granulated	Ambient
Lipases				
Cp-lipase	PhylloZyme	*Mycobacterium tuberculosis*	Powder	Ambient
Lipase LP-10	Jiangsu Boli Bioproducts	*Aspergillus niger*	Powder	4 °C
Lipase LP-100L	Jiangsu Boli Bioproducts	*Aspergillus niger*	Liquid	4 °C
Lipase NL-GX	Enzyme Supplies	*Aspergillus* sp.?	Liquid	4 °C
Alkaline Lipase	Creative Enzymes	*Aspergillus* sp.?	Granulated	Ambient
Mannanases				
Cp-mannanase	PhylloZyme	*Trichoderma reesei*	Powder	Ambient
Mannaway	Novozymes	*Bacillus* sp.?	Granulated	Ambient

Comparative evaluation of crude leaf extracts stability with commercial enzymes

Stability of leaf enzyme in plant lyophilized powder stored at ambient temperature for >10–12 months was evaluated in three independent biological samples of crude leaf extracts, with or without protease inhibitors. Total soluble protein (TSP) from the plant powder was extracted in the absence of protease inhibitors cocktail (PIC) and run on the SDS-PAGE. The presence of intact bands of proteins visualized on the Coomassie-stained gel indicated their stability (Figure 3a). However, direct proof of protein/enzymes stability should be evaluated through measurement of enzyme activity. In the absence of protease inhibitors, crude extract of Cp-mannanase, Cp-Eg1 and Cp-CelD showed equivalent activity while Cp-lipase showed 20%–25% higher activity when compared to extract with protease inhibitors. (Figure 3b). These results suggest stability of enzymes in liquid without protease inhibitors, in sharp contrast to commercial products that require formulation to stabilize enzymes and/or purification.

Protein extracts of Cp-Eg1 (LAMD) and Cp-lipase (LAMD and TN90) were also compared with commercial products through the Coomassie-stained SDS-PAGE gel (Figure 3a). In SDS-PAGE, TSP of Cp-Eg1 and Cp-lipase from lyophilized plant cells showed polypeptides of molecular mass ~25 and ~40 kDa, respectively, but not in untransformed WT plants. All tested microbial cellulase enzymes/products are highly concentrated except Bioprime® LX-1002 (Biogreen) & Cellulase G-CL (Enzyme Supplies) and showed several polypeptides (Figure 3a). The difference in size of polypeptides in microbial products could be attributed to different sources (origin) of enzymes based on predicted molecular mass. Acid cellulase (Sinobios) and Cellulase AC-100 (Jiangsu Boli Bioproducts) showed three prominent polypeptides in ~38 to ~70 kDa range. Similarly, Cellulase ACx (Enzyme supplies) and Cellulase NC-100 (Jiangsu Boli Bioproducts) displayed polypeptides of ~35 kDa to ~55 kDa in size. Cellusoft® L (Novozymes) and Bioprime® LX-1002 showed ~30 kDa proteins. Similarly, band intensity of all three tested commercial lipase LP-10, LP-100L (Jiangsu Boli Bioproducts) and Lipase NL-GX (Enzyme Supplies) was several folds higher when compared to Cp-lipase (LAMD) and Cp-lipase (TN90). These microbial products are protected by stabilizing agent with protease inhibitors or microbial proteases have been eliminated through expensive purification processes as revealed by their absence. It is evident that Cp-Eg1 and Cp-lipase are functional at lower concentrations than commercial enzymes. Exact quantity of commercial products could not be determined by densitometry because of multiple polypeptides identified in most of the commercial products. Moreover, quantification through Western blot was not done due to non-availability of specific antibody against all commercial products and inadequate details of enzyme source (genus, species, strain) in commercial products.

Biowashing of desized denim fabric using crude leaf extracts or commercial enzymes

Denim biowashing experiments were performed in 25-mL beaker following current industrial standards for 1 h at 60 °C for Cp-CelD and 50 °C for Cp-Eg1. The visual pepper-salt effects of Cp-Eg1 or Cp-CelD on denim biowashing was evaluated and compared to the effects observed with Novoprime 868, which includes both endoglucanase activity and exoglucanase activity. Crude leaf extract of Cp-Eg1 or Cp-CelD showed uniform removal of indigo dye from denim surface without compromising fabric quality (Figure 4a,b). However, commercial Novoprime 868 showed uneven patchy dye removal after biowashing (Figure 4a) or lesser dye removal (Figure 4b), with comparable enzyme units

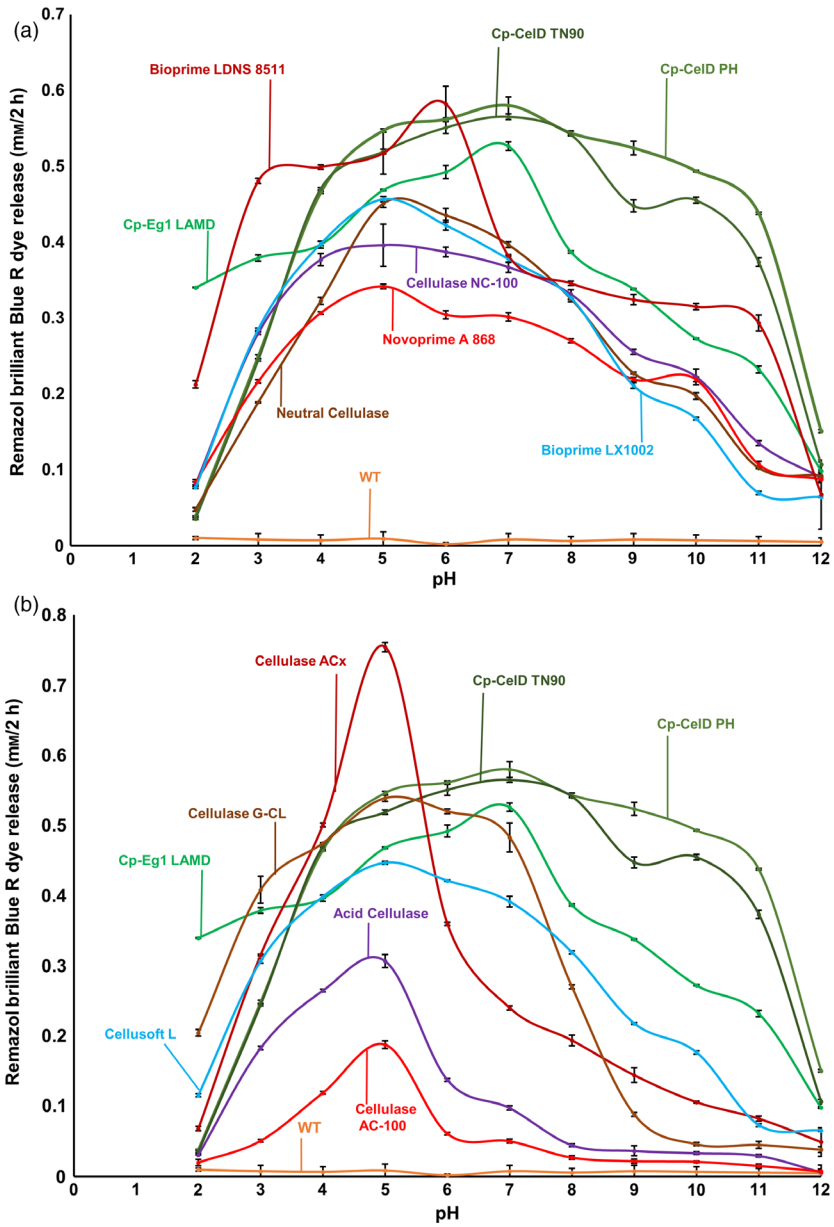

Figure 1 (a) Comparison of pH optima of endoglucanases in leaf crude extracts (Cp-CelD and Cp-Eg1) with commercial microbial enzymes. Lyophilized powder (10 mg) of Cp-CelD (PH), Cp-CelD (TN90) and Cp-Eg1 (LAMD) was extracted in 100 μL sodium acetate buffer (50 mM). Commercial enzymes (100 μL) with suitable dilutions were used. The assay was performed using 2% azo-CMC substrate in 50 mM sodium acetate buffer at 60 °C for Cp-CelD (PH) and Cp-CelD (TN90); 50 °C for Cp-Eg1 (LAMD). WT untransformed plant is used as the negative control. Commercial enzymes: Bioprime® LX-1002 (Biogreen), Bioprime® LDNS 8511(Biogreen), Novoprime A 868® (Novozymes), Cellulase NC-100 (Jiangsu Boli Bioproducts) and Neutral Cellulase (Sinobios). Enzyme assays were performed in three independent biological samples, and data present the average and standard deviation. (b) Comparison of pH optima of endoglucanases from leaf crude extracts (Cp-CelD and Cp-Eg1) with commercial microbial enzymes. Lyophilized powder (10 mg) of Cp-CelD (PH), Cp-CelD (TN90) and Cp-Eg1 (LAMD) was extracted in 100 μL sodium acetate buffer (50 mM). Commercial enzymes (100 μL) with suitable dilutions were used. The assay was performed using 2% azo-CMC substrate in 50 mM sodium acetate buffer at 60 °C for Cp-CelD (PH) and Cp-CelD (TN90) and 50 °C for Cp-Eg1 (LAMD). WT untransformed plant is used as the negative control. Commercial enzymes: Acid Cellulase (Sinobios), Cellulase AC-100 (Jiangsu Boli Bioproducts), Cellusoft® L (Novozymes), Cellulase G-CL (Enzyme Supplies) and Cellulase ACx 8000L/8000P (Enzyme Supplies). Enzyme assays were performed in three independent biological samples, and data present the average and standard deviation.

of purified microbial enzyme or plant crude extracts. Cp-Eg1, Cp-CelD and Novoprime 868 showed comparable endoglucanase activity when analysed in three independent samples before or after the biowashing experiments (Figure 4a,b). While it is not surprising that purified, concentrated and formulated commercial enzyme is stable, endoglucanase stability in crude leaf extracts without any protease inhibitors was not anticipated and offers unique cost advantages.

Biopolishing of knitted fabric using crude leaf extracts or commercial enzymes

The efficiency of biopolishing of knitted cotton fabric with crude leaf extract (Cp-Eg1, and Cp-CelD) and Cellusoft L (Novozymes) was done at 50 °C in pH 5.5 and evaluated by scanning electron micrographs (SEM) (Figure 4c,d). Both Cp-Eg1 and Cp-CelD were as efficient as commercial Cellusoft enzyme in depilling of knitted fabric (Figure 4c,d; upper panels) as observed by removal of horizontal strands in SEM. No biopolishing was observed in the negative control with a dense network of horizontal strands as indicated with arrow in figures (Figure 4c,d; upper panel). Reducing sugar endoglucanase assay showed slightly higher activity of Cp-CelD when compared to Cellusoft at the end of biopolishing when started with equivalent dosing of enzymes (Figure 4d; lower panel). Cp-CelD crude leaf extracts unprotected from proteases and purified commercial enzymes showed similar stability during biopolishing experiments (Figure 4d). However, Cp-Eg1 enzyme showed 50% reduction in activity at the end of biopolishing experiment (Figure 4c; lower panel).

Stain removal by detergents with crude leaf extracts or commercial enzymes

Chocolate destaining experiments performed at 30 °C showed visual destaining effect by both Cp-mannanase and Mannaway, when compared to the detergent only controls. We observed higher reflectance in the destained fabric with Cp-mannanase than Mannaway, with the highest reading at 450 nm (Figure 5a; upper panel). Chocolate stain removal at 70 °C was observed only with Cp-mannanase (Figure 5a; lower panel)

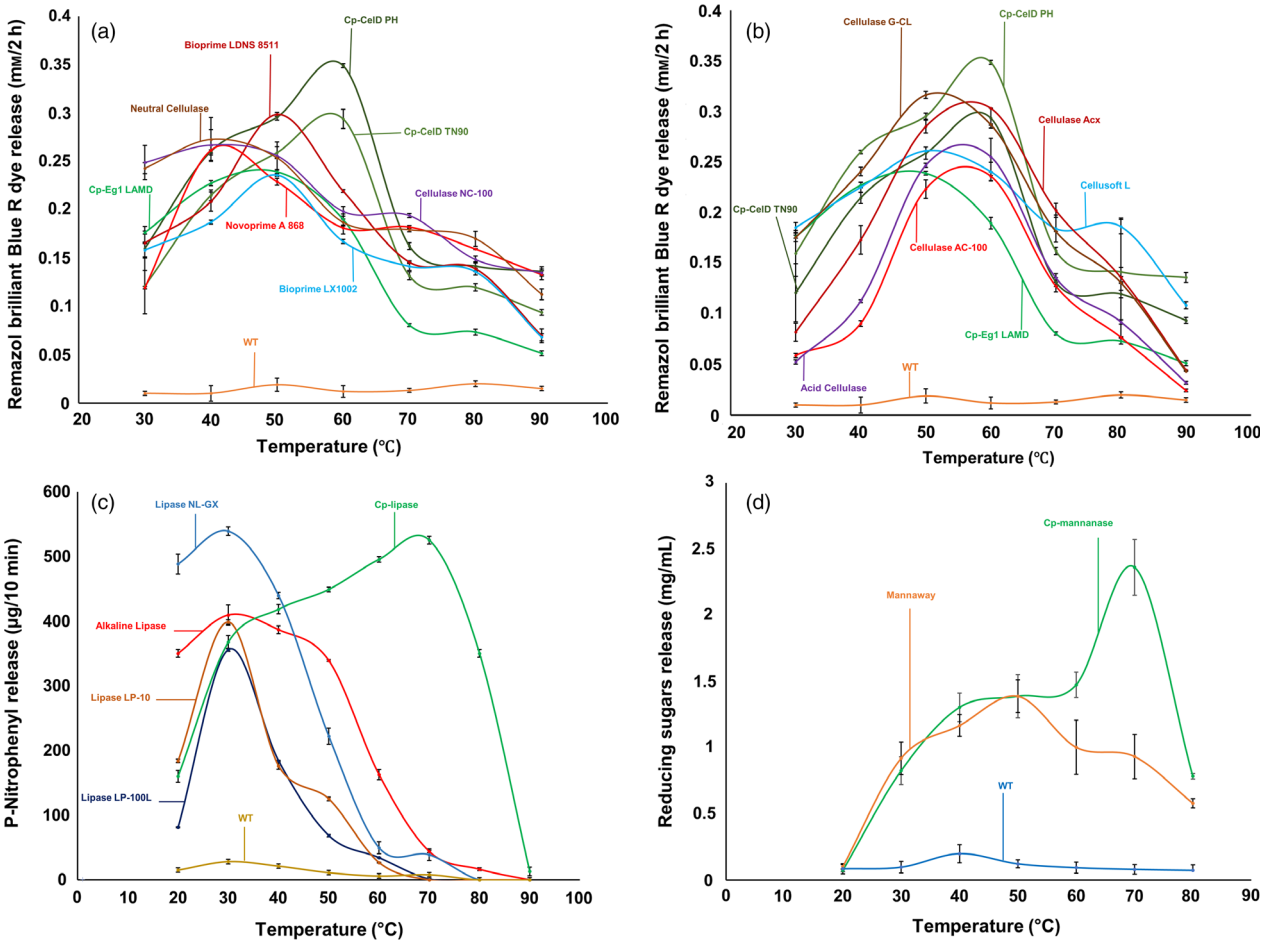

Figure 2 (a) Comparison of temperature optima of endoglucanase from leaf crude extracts (Cp-CelD and Cp-Eg 1) with commercial microbial enzymes. Lyophilized powder (10 mg) of Cp-CelD (PH), Cp-CelD (TN90) and Cp-Eg1 (LAMD) was extracted in 100 µL sodium acetate buffer (50 mM, pH 5.5). Commercial enzymes (100 µL) with suitable dilutions were used. The assay was performed using 2% azo-CMC substrate in 50 mM sodium acetate buffer. WT untransformed plant is used as the negative control. Commercial enzymes: Bioprime® LX-1002 (Biogreen), Bioprime® LDNS 8511(Biogreen), Novoprime A 868® (Novozymes), Cellulase NC-100 (Jiangsu Boli Bioproducts) and Neutral Cellulase (Sinobios). Enzyme assays were performed in three independent biological samples, and data present the average and standard deviation. (b) Comparison of temperature optima of endoglucanase from leaf crude extracts (Cp-CelD and Cp-Eg1) with commercial microbial enzymes. Lyophilized powder (10 mg) of Cp-CelD (PH), Cp-CelD (TN90) and Cp-Eg1 (LAMD) was extracted in 100 µL sodium acetate buffer (50 mM, pH 5.5). Commercial enzymes (100 µL) with suitable dilutions were used. The assay was performed using 2% azo-CMC substrate in 50 mM sodium acetate buffer. WT untransformed plant is used as the negative control. Commercial enzymes: Acid Cellulase (Sinobios), Cellulase AC-100 (Jiangsu Boli Bioproducts), Cellusoft® L (Novozymes), Cellulase G-CL (Enzyme Supplies) and Cellulase ACx 8000L/8000P (Enzyme Supplies). Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation. (c) Temperature optima of Cp-lipase from crude leaf extract and commercial microbial lipases. Lyophilized leaf powder (10 mg) was extracted in 100 µL sodium phosphate buffer (50 mM, pH 8). All reactions were performed at pH 8, 20-90 °C using p-Nitrophenyl butyrate (pNPB) as substrate, and pNP released in 10 min was measured. Commercial lipases; LP-100L, LP-10® (Jiangsu Boli Bioproducts), Alkaline Lipase® (Creative Enzymes) and Lipase NL-GX® (Enzyme Supplies) of 50 µL with suitable dilutions were used in assay. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation. (d) Temperature optima of Cp-mannanase from crude leaf extract and commercial microbial Mannaway. Lyophilized leaf powder (10 mg) was extracted in 100 µL of sodium citrate buffer (50 mM, pH 5). All reactions were performed using locust bean gum (LBG) as substrate in 50 mM sodium citrate buffer pH 5, and temperature ranging from 20 to 80 °C. Commercial Mannaway (100 µL) with suitable dilutions was used in assay. Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.

by visual observation or reflectance analysis, even though much higher stain intensity was tested. However, no peak at 450 nm was observed in any sample, probably because of heavy back staining at 70 °C. Back staining by mannans is possibly because of mannan Glue effect, that is particulate stain/soils released during the wash cycle binding to invisible residual mannan that may result in reappearance of the stain or fabric greying (Dhawan and Kaur, 2007). Release of sugars complicated measurements with increased background in DNSA method and therefore mannanase activity at the conclusion of experiment could not be performed. These results reflect data observed in enzyme temperature optima experiments (Figure 2d).

Mustard oil stain removal at 30 °C showed visually better clearance by both Cp-lipase and LP-10 lipase, when compared to detergent only control. High reflectance was observed by Cp-lipase crude extract and LP-10 lipase when compared to

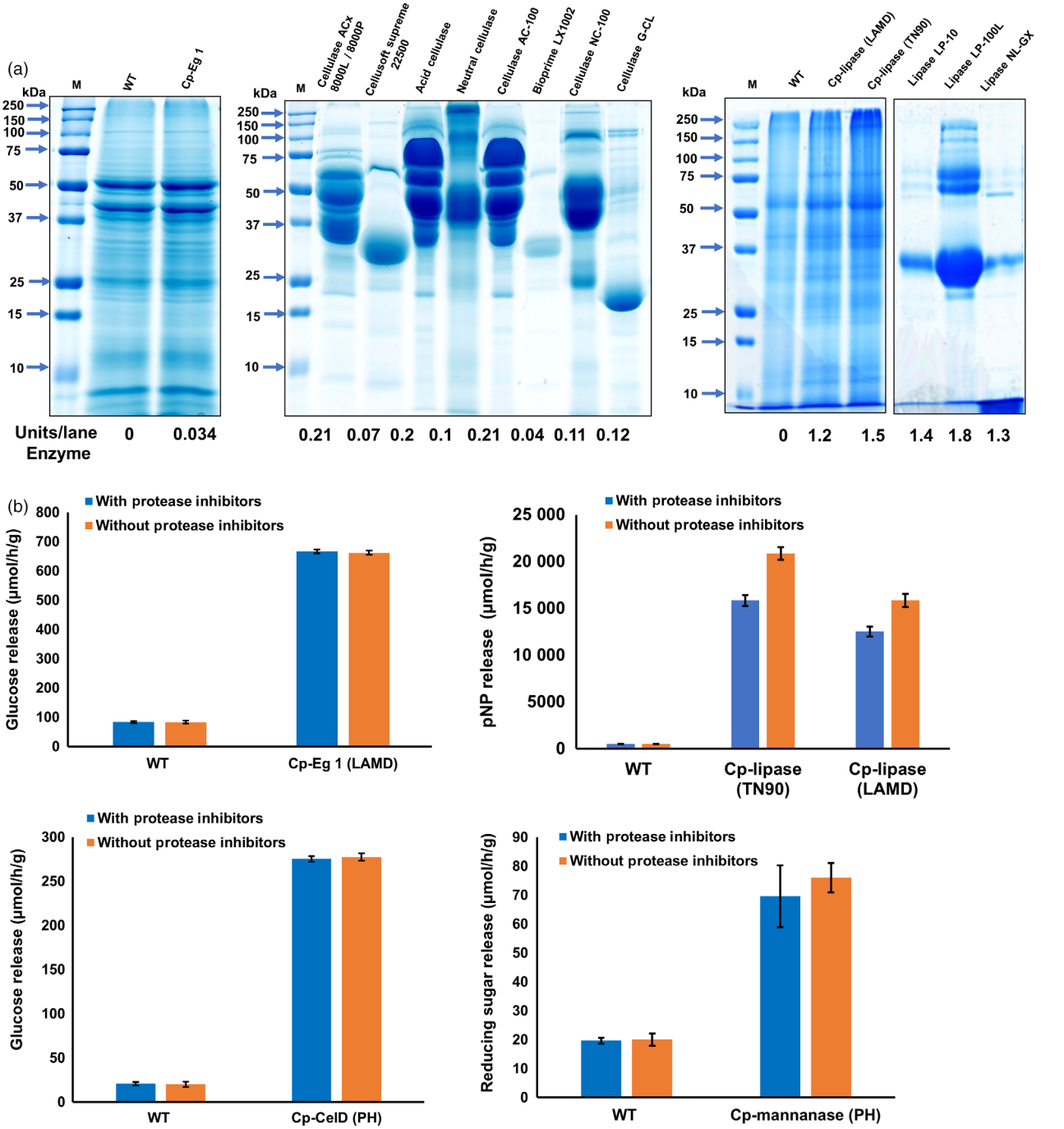

Figure 3 Evaluation of protein profile and stability of Cp-Eg1 and Cp-lipase leaf crude extracts with commercial microbial-enzyme products. (a) SDS-PAGE stained with Coomassie Brilliant blue. Lyophilized powder of Cp-Eg1 and Cp-lipase was extracted in respective buffer, and ~15 µg Cp-Eg1 and ~12 µg Cp-lipase TSP were loaded. WT untransformed plant is used as the negative control. Commercial products (1 µL) loaded for Cellulase ACx 8000L/8000P (Enzyme Supplies), Cellusoft® L (Novozymes), Acid Cellulase (Sinobios), Neutral Cellulase (Sinobios), Cellulase AC-100 (Jiangsu Boli Bioproducts), Bioprime® LX-1002 (Biogreen), Cellulase NC-100 (Jiangsu Boli Bioproducts), and Cellulase G-CL (Enzyme Supplies). Enzyme units equivalent to Cp-lipase were loaded for commercial Lipase LP-100L, LP-10® (Jiangsu Boli Bioproducts) and Lipase NL-GX® (Enzyme Supplies). (b) Graph shows Endoglucanases (Cp-Eg1, Cp-CelD), Cp-mannanase and Cp-lipase activity performed with leaf crude extract (extracted with and without Protease inhibitor cocktail (PIC)). Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.

detergent only control. To evaluate correlation between stain removal and enzyme activity, lipase assays against pNPB at 30 °C were also performed. Comparable lipase activity was observed at the start of this experiment while LP-10 lipase and Cp-lipase showed 42% and 25% lipase activity reduction at the end of stain removal experiment (Figure 5b; upper panel). When mustard oil destaining experiment was performed at 70 °C, visually better clearance of destained cloth was obtained by the Cp-lipase

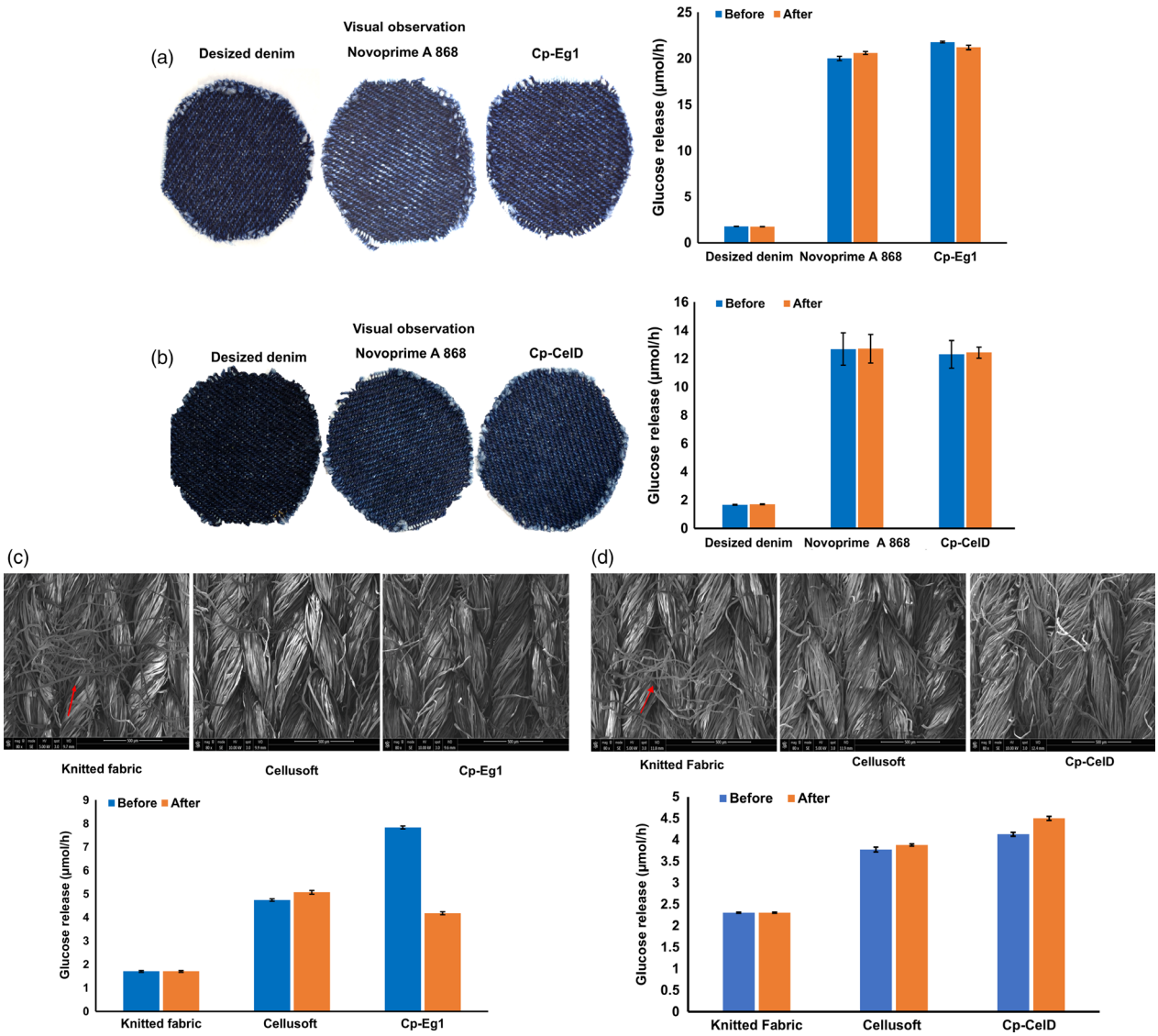

Figure 4 (a) Biowashing of denim fabric with Cp-Eg1 leaf crude extracts or Novoprime A 868® (Novozymes). Experiments were performed in 25 mL buffer (pH 5.5) in beakers at 50 °C for 1 h with desized denim fabric, Novoprime A 868 and crude leaf extract expressing Cp-Eg1. Graph shows endoglucanase activity performed before and after denim biowashing using 0.5% CMC-Na as substrate. Dinitrosalicylic acid was used to estimate reducing sugars released during assay. Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation. (b) Biowashing of denim fabric with Cp-CelD leaf crude extracts or Novoprime A 868® (Novozymes). A 25-mL beaker with magnetic stir bar was setup for 1 h at 60 °C with desized denim fabric, Novoprime A 868 and crude leaf extract expressing Cp-CelD. Graph shows enzyme activity before and after denim wash experiment using 0.5% CMC-sodium as substrate and dinitrosalicylic acid to estimate released sugars. Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation. (c) Biopolishing of knitted fabric with Cp-Eg1 leaf crude extracts or Cellusoft L (Novozymes). Upper panel shows scanning electron micrographs of the knitted fabric treated with Cellusoft (Novozyme) or Cp-Eg1 crude leaf extract in 25 mL sodium acetate buffer (50 mM, pH 5.5) at 50 °C for 1 h. Knitted fabric incubated with 25 mL sodium acetate buffer (50 mM, pH 5.5) served as a negative control. Graph in lower panel shows enzyme activity before and after biopolishing experiment. CMC-sodium (0.5%) was as substrate and dinitrosalicylic acid to estimate released sugars. Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation. (d) Biopolishing of knitted fabric with Cp-CelD leaf crude extracts or Cellusoft® L Novozymes). Upper panel shows scanning electron micrographs of knitted fabric treated with Cellusoft (Novozyme) or Cp-CelD crude leaf extract in 25 mL sodium acetate buffer (50 mM, pH 5.5) at 60 °C for 1 h. Knitted fabric incubated with 25 mL sodium acetate buffer (50 mM, pH 5.5) served as a negative control. Graph in lower panel shows enzyme activity before and after biopolishing experiment. CMC-sodium (0.5%) was as substrate and dinitrosalicylic acid to estimate released sugars. Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation.

crude extract, while LP-10 and detergent only control showed poor stain removal. Similarly, the high reflectance (450 nm peak) was observed in destained cloth treated with Cp-lipase crude extract. Estimated lipase activity of Cp-lipase at the start of stain removal experiment at 70 °C was two times higher than the LP-10 lipase. Moreover, Cp-lipase activity was four times higher than commercial lipase at the end of the stain removal experiment (Figure 5b; lower panel).

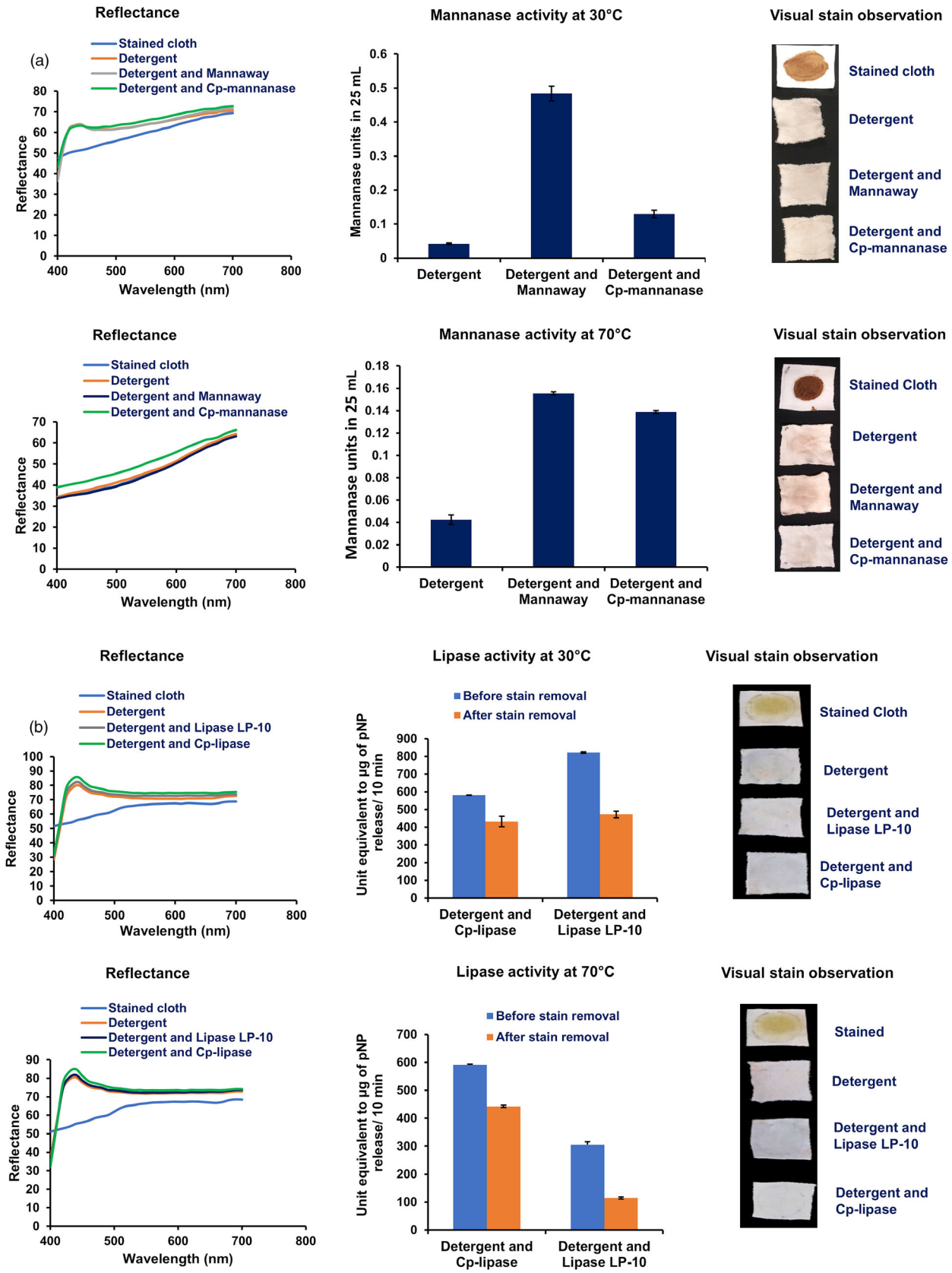
Reflectance
(a)
Stained cloth
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Reflectance
Wavelength (nm)
Mannanase activity at 30°C
Mannanase units in 25 mL
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Visual stain observation
Stained cloth
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Reflectance
Stained cloth
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Reflectance
Wavelength (nm)
Mannanase activity at 70°C
Mannanase units in 25 mL
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Visual stain observation
Stained Cloth
Detergent
Detergent and Mannaway
Detergent and Cp-mannanase
Reflectance
(b)
Stained cloth
Detergent
Detergent and Lipase LP-10
Detergent and Cp-lipase
Reflectance
Wavelength (nm)
Lipase activity at 30°C
Before stain removal
After stain removal
Unit equivalent to µg of pNP release/ 10 min
Detergent and Cp-lipase
Detergent and Lipase LP-10
Visual stain observation
Stained Cloth
Detergent
Detergent and Lipase LP-10
Detergent and Cp-lipase
Reflectance
Stained cloth
Detergent
Detergent and Lipase LP-10
Detergent and Cp-lipase
Reflectance
Wavelength (nm)
Lipase activity at 70°C
Before stain removal
After stain removal
Unit equivalent to µg of pNP release/ 10 min
Detergent and Cp-lipase
Detergent and Lipase LP-10
Visual stain observation
Stained
Detergent
Detergent and Lipase LP-10
Detergent and Cp-lipase

Figure 5 (a) Chocolate stain removal by Cp-mannanase and commercial Mannaway. Enzymes used as additive in laundry detergent and performance evaluated by measuring reflectance of fabric by SS5100H dual beam spectrophotometer (Premier Colorscan Instruments), mannanase activity of the enzymes and visual effect of destained fabric. Stain removal was performed in 25 mL buffer with detergent and respective enzyme for 30 min at 30 °C (Upper panel) and 70 °C (Lower panel). Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation. (b) Mustard oil stain removal by Cp-lipase and commercial lipase LP-10 (Jiangsu Boli Bioproducts). Enzymes used as additive in laundry detergent and performance evaluated by measuring reflectance of fabric by SS5100H dual beam spectrophotometer (Premier Colorscan Instruments), lipase activity before and after destaining by the enzymes and visual effect of destained fabric. Stain removal was performed in 25 mL water with detergent and respective enzyme for 30 min at 30 °C (Upper panel) and 70 °C (Lower panel). Enzyme assays were performed in three aliquots from each beaker, and data represent the average and standard deviation.

Marker-free lettuce chloroplasts expressing different enzymes

The marker-free chloroplast vector (pLsLF-MF) was constructed by sequential cloning steps containing aminoglycoside-3′-adenylyl-transferase (*aadA*), the spectinomycin resistant gene under the control of plastid ribosomal RNA promoter (*Prrn*) and followed by 3′UTR, *TrbcL*. The *aadA* gene is located between two copies of 649-bp direct repeats of chloroplast-encoded CF1 ATP synthase subunit beta (*atpB*) promoter region (Figure 6a,b; upper left panel). A homologous recombination process between the 649-bp direct repeats should loop out the *Prrn*, *aadA* and *TrbcL* fragment. The coding sequences of *LipY*, *Cbh1*, *Cbh2* genes were inserted/cloned into pLsLF-MF, under the control of the *psbA* promoter/5′ UTR and 3′ UTR (Figure 6a,b). After 4–6 weeks of bombardment, the leaf explants showed direct shoot development, without an intervening callus phase on spectinomycin-containing selection medium. During the selection process on spectinomycin-containing RMOP medium, the *aadA* cassette is excised, releasing one copy of the *atpB* region in the transplastomic genome by homologous recombination between the two directly repeated *atpB* fragments. After homologous recombination-based marker excision, 16s-Forward/atpB-Reverse primers amplified the 2.4 kb PCR product (Figure 6a; lower panel left, Lanes 1,2, 3, 4, 5 and 6b; lower panel left, Lanes 1–6) and the 16s-Forward/aadA-Reverse did not produce any amplified PCR product (lower middle panel of Figure 6a,b). If the transplastomic genome contained the intact expression cassette with *aadA* gene, a 4.4 kb DNA fragment should be produced in addition to the 2.4 kb product. Site-specific integration of expression cassette at the 3′ end is confirmed by a 2.2 kb PCR product amplfied by the UTR-F/23s-R (Figure 6a Lanes 1, 2, 3, 5; Figure 6b Lanes 1–6 lower panel).

Southern blot analysis was carried out to confirm site-specific integration homoplasmy and removal of the marker gene (*aadA*) from the expression cassette of *LipY, Cbh1 or Cbh2* genes into chloroplast genome. In Southern blot, ~3.1 kb fragment and ~5.6 or 6 kb fragment were expected for untransformed plant (WT) and transplastomic lines respectively, when digested with *Sma*I/*Xma*I and hybridized with *trnI-trnA* flanking sequence 1.1 kb probe (Figure 6a and b; top right panel). All independent transplastomic lines of *LipY, Cbh1 or Cbh2* showed distinct hybridizing band with expected size of ~5.6 and ~6 kb respectively, after removal of marker gene except in 3 and 3#2 transplastomic line, which showed no integration of *LipY* gene or excision of whole expression cassette at the time of marker removal (Figure 6a; top right panel).

PCR and Southern blot analysis of lettuce plants showed homoplasmy with site-specific integration of transgene expression cassettes containing *LipY, Cbh1 or Cbh2* genes in all tested lines (Except *LipY* 3, 3#2 and 4) and removal of the *aadA* gene. Recombinant expression of lipase was also confirmed from *in vitro* grown marker-free lettuce plants by activity assay (Figure 6a; bottom right panel).

Large-scale biomass production and enzyme yield

Transplastomic plants were grown at commercial scale in the greenhouse or hydroponic Fraunhofer production system. Biomass was harvested from the plants when the leaf was fully mature and total 5 times leaf biomass were harvested from Fraunhofer except for Cp-mannanase 4 times (Figure 7a,b). Cp-mannanase plants initially grew slowly, but at the later stage, biomass yield per plant was comparable to other enzyme producing plants. For Cp-mannanase, biomass yield in Fraunhofer increased consistently from 3.2 to 9.8 g FW/plant as their age increased from 9.5 to 24.5 weeks (Figure 7b; upper left panel). Cp-mannanase greenhouse grown plant biomass yield increased from 78.2 to 90 g FW/plant as their age increased from 6 to 10.5 weeks and became constant (~90 g FW/plant) at later harvests (Figure 7b; upper right panel). Biomass yield in the greenhouse was 23- to 28-fold higher than Fraunhofer at different stages of leaf harvest. Cp-mannanase enzyme activity from greenhouse grown plants was similar (~3.4 μmol/h/g FW) for each harvest, while Fraunhofer grown plants had similar mannanase activity (4.8–5.5 μmol/h/g FW) in different harvests (Figure 7b; lower right panel). Cp-mannanase greenhouse grown plants showed lower enzyme activity (1.3- to 1.7-fold) at different stages of leaf harvest when compared to Fraunhofer.

Cp-lipase (LAMD) plants grown in the greenhouse yielded 61.9–106.0 g FW/plant during 6–15 weeks. Cp-lipase (TN90) plant biomass yield increased from 49.6 to 139.1 g FW from 6 to 15 weeks (Figure 7b; upper left panel). Fraunhofer Cp-lipase (TN90), biomass yield increased from 5 to 11.4 g FW/plant in 6.5 to 24.5 weeks (Figure 7b; upper right panel), Cp-lipase (LAMD) biomass yield increased from 7.3 to 13.3 g FW/plant in 6.5 to 24.5 weeks (Figure 7b; upper right panel). Fraunhofer biomass yield per plant was inconsistent in all harvests. Comparison of greenhouse and Fraunhofer, Cp-lipase (LAMD) and Cp-lipase (TN90) biomass yield in the greenhouse was 8- to 20-fold and 10- to 36-fold higher in each harvest, respectively. Cp-lipase (TN90) showed almost similar enzyme activity (~171 μmol/h/g FW) at each harvest from Fraunhofer, while in the greenhouse increased from 79 to 98 μmol/h/g FW in 7.5 to 15.5 weeks (Figure 7b; lower left panel). In Cp-lipase (LAMD), similar enzyme activity (~177 μmol/h/g FW) was obtained in greenhouse grown plants, while in Fraunhofer 59–94 μmol/h/g FW activity was observed from 6.5 to 12 weeks (Figure 7b; upper lower panel). Cp-lipase (LAMD) enzyme activity in greenhouse grown plants was 2- to 3-fold higher than Fraunhofer, whereas Cp-lipase (TN90) was 1.7- to 2.1-fold lower in all harvests.

Biomass yield of Fraunhofer grown Cp-CelD per plant decreased from 6.04 g in 6 weeks to 4.4 g FW/plant in 9 weeks

for PH cultivar but increased from 6.3 to 8.6 g FW/plant for TN90 cultivar. In contrast, the biomass yield increased from 55.4 g in 6 weeks to 129.6 g FW/plant in 9 weeks in the greenhouse grown Cp-CelD PH plants (Figure 7a; upper panel). Cp-CelD TN90 cultivar showed maximum enzyme activity (26–33 µmol/h/g FW) in Fraunhofer grown plants, and Cp-CelD PH cultivar showed comparable activity in both greenhouse and Fraunhofer, except at 9.5 weeks (Figure 7a; lower panel). Similarly, Cp-Eg1 (LAMD)

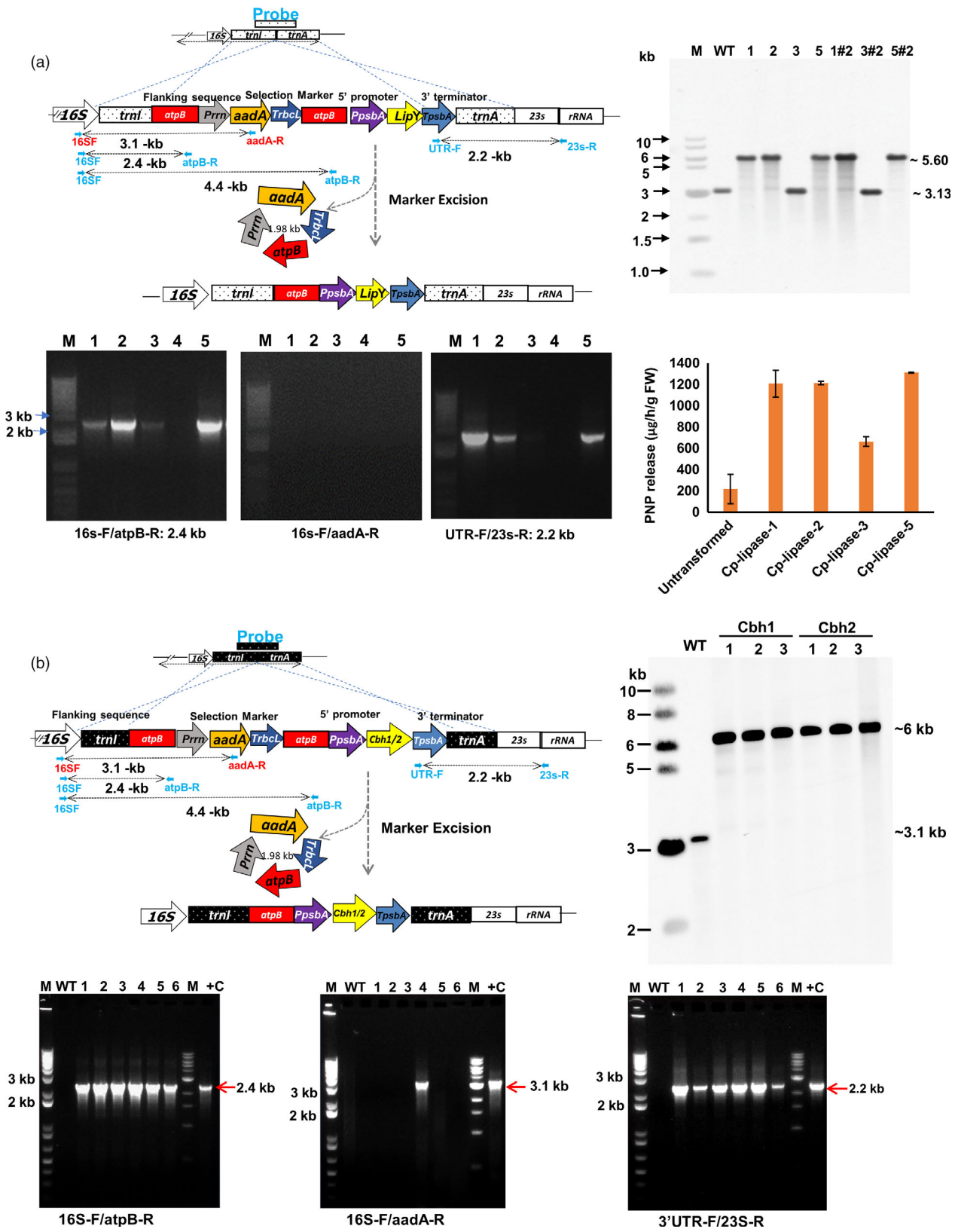

Figure 6 (a) Molecular analysis of Marker-free lettuce transplastomic lines expressing lipase (*lipY*). Top left panel: Schematic representation of the lettuce chloroplast 16S *trnI / trnA* region, chloroplast expression cassette containing lipase (*lipY*) transgene cassette. Bottom left panel: PCR analysis of transplastomic lines using 16S-F/atpB-R, 3′UTR-F/23s-R and 16S-F/aadA-R primer sets; PCR product of size 2.439 kb with primer set 16S-F/atpB-R and 2.454 kb with 3′UTR-F/23s-R confirms integration of cassette into the lettuce chloroplast genome; absence of PCR product with 16S-F/aadA-R primer sets confirms removal of marker gene from the transplastomic plants. Bottom right panel: Functional lipase in transplastomic lines evaluated in sodium phosphate buffer pH 8.0 in three independent biological samples and released pNP was measured at 400 nm. Top right panel: Southern blot hybridized with DIG-labelled *trn*I-*trn*A flanking sequence probe; presence of single band of size ~5.6 kb in transplastomic line 1, 2, 5, 1#2, 5#2 confirms integration of *Lip*Y gene and removal of antibiotic resistance gene, and all five are homoplasmic in nature. Band size of ~3.1 kb obtained in untransformed plant (WT) and transplastomic line 3 and 3#2 shows absence of *Lip*Y gene in these plants. In putative transplastomic line 3, initial screening by PCR and enzyme assay showed integration of gene in the chloroplast genome but absence of desired band size in Southern blot confirms the removal of entire expression cassette. (b) Molecular analysis of Marker-free lettuce transplastomic lines expressing Cellobiohydrolases (*Cbh1, Cbh2*). Top left panel: Schematic representation of the lettuce chloroplast 16S *trnI / trnA* region, chloroplast expression cassette containing *Cbh1, Cbh2* genes. Top right panel: Southern blot analysis confirms homoplasmy. Presence of ~3.1 and ~6 kb in untransformed plant (WT) and transplastomic line, respectively, confirms removal of antibiotic resistance *aadA* gene and integration of *Cbh1* or *Cbh2* gene into the lettuce chloroplast genome. Bottom panel: PCR product of size 2.439 kb with primer set 16S-F/atpB-R and 2.454 kb with 3′UTR-F/23s-R confirms integration of cassette into lettuce chloroplast genome; absence of PCR product with 16S-F/aadA-R primer sets confirms removal of marker gene from the transplastomic plants. Lane M: Marker, Lane WT: Untransformed plant, Lane +C: Positive control, previously confirmed transplastomic line. Lanes 1–3: *Cbh1* transplastomic lines: Lanes 4–6: *Cbh2* transplastomic lines.

biomass yield was 3.5 g in 6 weeks and increased to 8.85 g FW in 24 weeks in Fraunhofer (Figure 7a; upper panel). Cp-Eg1 (LAMD) and Cp-CelD (PH, TN90) greenhouse grown plants yielded 131, 129, 121 g FW/plant, respectively (Figure 7a, b, upper panel). In the greenhouse, observed FW biomass yield of Cp-Eg1 (LAMD) was 28-fold higher in 9 weeks when compared to Fraunhofer. The measured enzyme activity was higher for Cp-Eg1 grown in the greenhouse than Fraunhofer (Figure 7a; lower panel). Fraunhofer Cp-Eg1 plants showed a gradual increase in enzyme activity (44–67 μmol/h/g FW) with each harvest. Greenhouse grown Cp-Eg1 plants showed similar range of enzyme activity (84–95 μmol/h/g FW) in all harvests (Figure 7a; lower panel).

Discussion

Genetically engineered enzymes are produced in seeds taking advantage of ability to store at ambient temperature and ease of purification, but purified products require refrigeration for storage and transportation. However, no high-value products are produced in leaves, even though they could synthesize very high levels of proteins (Ngugi *et al.*, 2017). The chloroplast originated plant protein Rubisco is the most abundant protein on earth (Cosa *et al.*, 2001). Therefore, we explore here validation of high-value industrial enzymes made in tobacco or lettuce chloroplasts for detergent or textile applications as well as for edible food/feed applications. To the best of our knowledge, this is the first report of leaf-based commercial products for industrial applications (PhylloZyme leaf products – Figures S1, S2).

Chloroplasts are metabolic active centres for photosynthesis, for converting solar energy to carbohydrates. Chloroplast genetic engineering offers unique advantages including high-level expression of foreign proteins (Bally *et al.*, 2009; Clarke *et al.*, 2017; Daniell *et al.*, 2016a,b; Jin and Daniell, 2015; Sanz *et al.*, 2011), transgene containment through maternal inheritance (Daniell, 2002, 2007) and production in leaves enables harvest before flowering offering almost complete transgene containment. Currently, Fraunhofer hydroponic facility is used by PhylloZyme to produce biomass for enzyme products. However, biomass yield per plant was less in Fraunhofer than the greenhouse: 3- to 30-fold for Cp-CelD, 9- to 58-fold for Cp-Eg1, 10- to 36-fold for Cp-lipase (TN90), 8- to 20-fold Cp-lipase (LAMD) and 23- to 28-fold for Cp-mannanase for all harvests. Enzyme activity obtained for Cp-mannanase, Cp-CelD and Cp-lipase (TN90) enzymes in greenhouse was 1- to 2-fold less than Fraunhofer, but their biomass yield was 10- to 36-fold higher in greenhouse. Therefore, overall enzyme yield for all these enzymes from total biomass was significantly higher for the greenhouse grown plants. Biomass and enzyme activity data of greenhouse and Fraunhofer suggest that greenhouse will be the preferred choice for large-scale production of leaf enzymes.

Higher biomass production will be required for industrially important enzymes for food-feed, textile, brewery, detergent, paper, pulp, wastewater treatment, bioethanol and various other industrial application. Cost of enzymes for cellulosic ethanol varies between 15% and 25% of the biorefinery processing (Park *et al.*, 2016), limiting further advancement of this technology/concept. In order to meet the market demand, the large-scale leaf biomass production may require plant growth in open fields. USDA-APHIS approved field production of transplastomic line expressing IFN-α2b has been done almost a decade ago (Arlen *et al.*, 2007). According to USDA-APHIS notice (Kwon and Daniell, 2015), transplastomic lines do not fit the definition of a regulated article under USDA-APHIS regulations 7 CFR part 340, because there are no plant pest components, which should further help in advancing this technology. Considering low production cost of tobacco ($3213/acre, $1.48–1.85/lb cured leaf, Daniell *et al.*, 2019), it would be cheaper to produce enzymes in the field than any other current microbial production system.

In the present study, stability of proteins/enzymes in the leaf powder was achieved by the removal of water through freeze-drying process (lyophilization). This approach also alleviates the necessity of cold storage/transportation of leaf enzymes and has been used in the Daniell lab for biopharmaceuticals expressed in leaves (Daniell *et al.*, 2016a,b; Su *et al.*, 2015). However, lyophilizers are very expensive, require 3 days for total dehydration and have limited capacity, underscoring the need to develop alternative low-cost approaches. It has been reported that protein drugs produced in leaves are highly stable when plants were dried in the greenhouse, without watering (Boyhan and Daniell, 2011). Leaf drying at room temperature in sunlight is also reported for xylanase producing plants (Leelavathi *et al.*, 2003). Methods reported for processing of tea leaves to preserve quality and aroma by Chen *et al.*, 2019 could also be adopted. Freshly plucked tea leaves were naturally dried on bamboo sieves at 21–24 °C with 65%–82% humidity, for 48 h, and then further dehydrated at 60 °C for 2 h to obtain the final tea product.

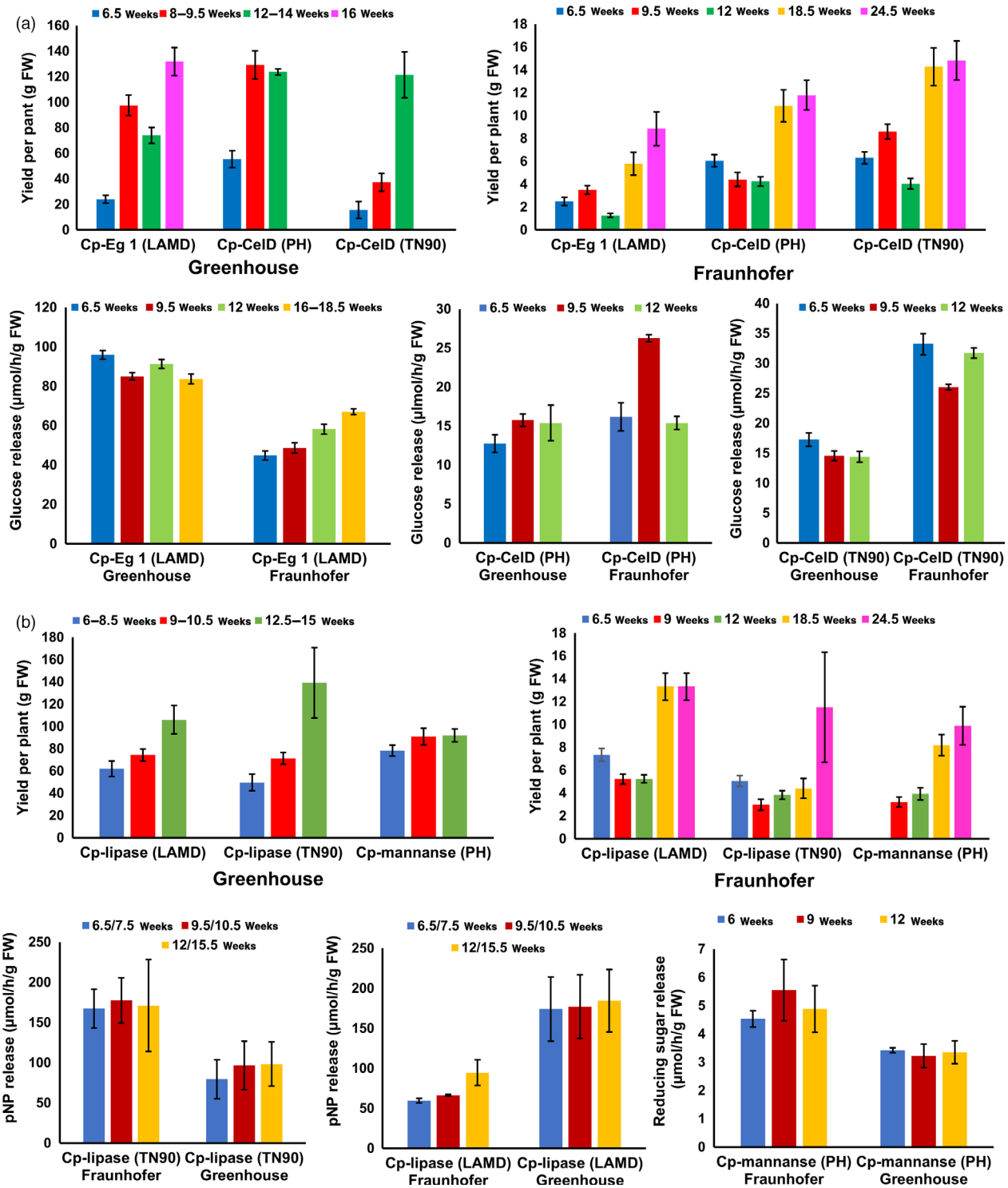

Figure 7 Biomass yield and enzyme activity of plant grown in hydroponic system at Fraunhofer or in the Daniell lab greenhouse. Biomass yield of tobacco plants grown at Fraunhofer and Daniell lab greenhouse expressing different enzymes in different cultivars (PH - Petit Havana), LAMD- (low nicotine) and TN90 (Upper panel). Comparative enzyme activity in Fraunhofer and greenhouse grown plants for the different harvest expressing Endoglucanase (Cp-Eg1, Cp-CelD), mannanase (Cp-mannanase) or lipase (Cp-lipases) (Lower Panel). (a) Cp-Eg1 (LAMD) and Cp-CelD (PH, TN90). (b) Cp-mannanase (PH) and Cp-lipase (LAMD, TN90). Enzyme assays were performed in three independent biological samples, and data represent the average and standard deviation.

In this study, we observed that enzymes present in lyophilized leaf biomass were stable at room temperature for several months because of total dehydration. Furthermore, leaf enzymes required no further purification or unique formulation for enzyme stability. In lyophilized leaves, enzymes were stable after storage for 10 (endoglucanases (Cp-Eg1, Cp-CelD)), 11–12 (Cp-lipases) and 10–12 (Cp-mannanase) months. However, the stability of Cp-CelD and Cp-lipase enzymes at higher temperature or pH during

biopolishing or biowashing is a functional characteristic of that specific protein and not their expression system. For example, Cp-CelD endoglucanase has much higher stability than Cp-Eg1 after biopolishing experiments at 50 °C in pH 5.5. Elimination of leaf-enzyme purification and formulation significantly decreases production cost because majority of cost (up to 90%) in any commercial product is attributed to downstream processing that involves purification and formulation.

The presence of different plant proteases in plant crude extracts did not affect chloroplast enzyme stability (Cp-Eg1, Cp-CelD, Cp-lipase, Cp-mannanase), and their activity was maintained after long incubations at alkaline pH or high temperatures, without addition of protease inhibitors. However, in the case of Cp-lipase, addition of protease inhibitors decreased its activity 20%–25%. The alleviation of necessity to add protease inhibitors in leaf enzymes makes production cost-effective. Except for Cp-Eg1, all crude leaf extracts (Cp-CelD, Cp-lipase) showed stability at the end of biopolishing, biowashing or strain removal experiments. Among all 19 commercial products analysed in the study, 10 products were in liquid and 9 were in powder/granulated formulation as listed in Table 1. All liquid enzymes were stored at 4 °C as recommended by manufacturers. Unfortunately, long-term storage resulted in microbial contamination. Among 9 solid formulation products, 3 were in granulated (Novoprime A 868, Mannaway, Alkaline Lipase) and 6 were in powder formulations. In all powder enzymes, only PhylloZyme products were stored at ambient temperature, while other powder form enzyme products (Lipase-10, Bioprime LDNS8511) were stored at 4 °C as recommended by manufacturers. Granulation done after purification of enzyme for formulation and stabilization is an expensive process, which is eliminated in leaf-powder enzymes made by PhylloZyme. Most strikingly, microbial commercial lipases and endoglucanases are highly concentrated than crude leaf extracts, when analysed on SDS-PAGE gels. This shows that purified commercial products are required in much higher concentrations when compared to enzymes/proteins produced in plants to carry out similar functions (Figure 3a).

Our study reports industrial validation of crude leaf extracts of Cp-Eg1 or Cp-CelD in denim biowashing and biopolishing. The textile industry requires cellulases which are active at neutral or alkaline pH with minimal back staining and improved fabric strength. The crude extracts of Cp-CelD showed a broad pH optimum (5–9) where they retained >90% activity. This broad pH range gives significant advantages in biowashing experiments. Denim industry faces significant problems due to high indigo back staining (Agrawal, 2017) at acidic pH due to high adsorption of enzyme on cellulosic fibres. With ~100% activity at pH 7, Cp-CelD and Cp-Eg1 extracts offer significant advantages over current commercial enzymes. In both biowashing and biopolishing studies, crude extracts of Cp-Eg1 or Cp-CelD showed comparable effects with commercial products (Novoprime 868 and Cellusoft supreme 22500). SEM images provide a qualitative confirmation of biopolishing with cellulases. Depilling or surface smoothening structural changes on cotton fabric occurred during biopolishing using cellulases were studied by SEM imaging (Arumugam *et al.*, 2007; Ibrahim *et al.*, 2011; Verenich *et al.*, 2008). Unlike Cp-CelD from *C. thermocellum* that maintained >90% activity at pH 9 or 100% activity at 70 °C, a sharp reduction (~50%) in Cp-Eg1 activity in biopolishing may be attributed to the gene source (*T. reesei*).

The present study shows that Cp-lipase and Cp-mannanase are suitable for their use in laundry detergents. Cp-lipase and Cp-mannanase enhanced the mustard oil and chocolate stain removal, when used as an additive in the laundry detergent. Better washing performance was confirmed by visual observation and increased reflectance of washed fabrics. Evaluation of surface reflectance of washed fabric in the visible range (400–700 nm) is the most common method to evaluate the cleaning performance for decades (Utermohlen and Ryan, 1949). Mustard oil stain removal property of Cp-lipase crude extract was similar to commercial microbial purified LP-10 lipase at 30 °C, while it was superior at 70 °C. Moreover, Cp-lipase higher performance for mustard oil removal at 70 °C was contributed by its higher thermostability. Therefore, leaf powder containing crude Cp-lipase has great potential as additive in the detergent industry to remove oil stain in broad temperature or pH range.

Chocolate stain removal property of crude Cp-mannanase was on par with commercial microbial purified Mannaway at 30 °C while Cp-mannanase was far better in stain removal at 70 °C. Both enzymes were stable in the presence of detergent/denaturants. Cp-mannanase chocolate stain removal efficiency at 70 °C was far superior than Mannaway because Cp-mannanase is a highly thermostable enzyme. Laundry detergent used in this study has no phosphate as water softener. Phosphate in modern detergent is not recommended due to environmental considerations/legislations. Formulation of Cp-mannanase and Cp-lipase in phosphate-free detergent is another important advantage.

Lipase and cellobiohydrolase expressed in high biomass producing leafy food crop (lettuce) are suitable for food applications. Moreover, excision of the antibiotic resistance genes from transplastomic crops not only reduces metabolic load but also provides the feasibility to use the same selection marker for subsequent transformation of additional genes. All genetically modified (GM) crops approved by the Food and Drug Administration (FDA) carry antibiotic resistance genes, and there are no antibiotic-free GM crops. However, GM transplastomic lines with antibiotic resistance gene may hinder in the regulatory approval process because of large gene copy numbers per cell. Daniell's lab has recently developed the expertise of marker-free approach for heterologous protein expression via the lettuce chloroplast genome, following the method of direct repeat homologous recombination method developed by Day's group (Day and Goldschmidt-Clermont, 2011; Iamtham and Day, 2000; Kode *et al.*, 2006). The expression cassette containing the *LipY*, *Cbh1 and Cbh2* genes used 649 bp of two *atpB* promoter regions to promote marker gene excision from the lettuce chloroplast genome. After site-specific integration of transgene cassettes containing *LipY*, *Cbh1 and Cbh2* genes into lettuce chloroplast genome, antibiotic marker gene was eventually excised. In the pLs-MF expression cassette of *LipY*, *Cbh1 and Cbh2* genes with two copies 649 bp *atpB* promoter regions to accelerate excision of marker gene from the lettuce chloroplast genome. Transplastomic lettuce plants showed correct site-specific integration of transgene cassettes containing *LipY*, *Cbh1 and Cbh2* genes without antibiotic resistance gene. In this study, Southern blot analysis showed deletion of the entire *LipY* expression cassette in one transplastomic line during the excision of the marker gene. Therefore, it is important to confirm marker-free lines because absence of selection could lead to loss on transgene cassette. Transplastomic lines showed normal growth in the greenhouse with expression of recombinant lipase. The availability of marker-free edible crop with these enzymes offers the unique platform for advancing food/feed applications of enzymes without antibiotic resistance genes.

Experimental procedures

Transplastomic tobacco biomass and lyophilization

Detail protocol of transplastomic tobacco biomass production and lyophilization is discussed in (Daniell *et al.*, 2019).

Endoglucanase assay: temperature and pH optimum

Enzyme activity of crude leaf extracts (Cp-CelD PH, Cp-CelD TN90 and Cp-Eg1 LAMD) was assayed and compared with commercial microbial endoglucanases listed in Table 1. For preparation of leaf crude extracts, 10 mg of lyophilized leaf biomass was extracted in 100 μL sodium acetate buffer (50 mM pH 5.5) by sonication (5 s on and 10 s off cycle; 3 times). Optimum pH was determined in buffer at pH range 2–12 using 2% (w/v) azo-CMC as substrate at 60 °C (Cp-CelD) and 50 °C (for Cp-Eg1) for 2 h. For temperature optimum, the substrate was dissolved in sodium acetate buffer (50 mM, pH 5.5), and enzyme assay was performed at temperature (30–90 °C) for 2 h. Untransformed (WT) was used as a negative control. All assays were performed using three independent biological samples. Endoglucanase activity in leaf extracts and commercial enzymes was measured spectrophotometrically (Abs_{590}) by monitoring the release of dye from 2% (w/v) azo-CMC (Reyes-Sosa *et al.*, 2017).

Lipase assay and temperature optima

Cp-lipase (LAMD, TN90 cv.) was grown, leaves harvested, lyophilized and ground into powder form. Protein was extracted from lyophilized powder of Cp-lipase and WT in 100 mM sodium phosphate buffer pH 8, and TSP was quantified. In enzyme assay, 50 μL crude leaf extract was added into the 450 μL of 100 mM sodium phosphate buffer pH 8 having 0.9% NaCl and pre-incubated at 70 °C for 10 min. After that 5 μL of p-nitrophenyl butyrate (100 mM) mixed in the reaction mixture, incubated at 70 °C for 10 min. Reaction was stopped by incubating on ice for 10 min, and released p-nitrophenyl was measured at 400 nm. The temperature optimization of Cp-lipase and comparison with commercial enzymes carried out in similar way using three independent biological samples with incubation at 20–90 °C in sodium phosphate buffer pH 8. Tested commercial lipase enzymes were LP-100L (Jiangsu Boli Bioproducts), LP-10 (Jiangsu Boli Bioproducts), Alkaline Lipase (Creative Enzymes) and NL-GX (Enzyme Supplies).

Evaluation of leaf and commercial products on SDS-PAGE

Lyophilized leaf powder of Cp-lipase, Cp-Eg1 and WT was extracted in protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl, pH 8, 100 mM dithiothreitol, 400 mM Sucrose), and extracted protein was quantified by Protein Assay Dye (Bio-Rad, Hercules, CA) using BSA as standard. Total soluble protein (TSP) of Cp-lipase, Cp-Eg1 and commercial products were resolved on 12% SDS-PAGE and were stained with Coomassie Brilliant Blue R-250 dye.

Biowashing of desized denim fabric

The biowashing of desized denim fabric was performed with chloroplast-derived endoglucanases (Cp-Eg1, Cp-CelD) and Novoprime 868. Total soluble protein was extracted by sonication (5 s on and 1 min off cycle; 5 times) from lyophilized biomass (Cp-CelD, Cp-Eg1) in sodium acetate buffer (50 mM pH 5.5) 1:20 (w/v) ratio. The denim biowashing experiment was conducted in 25 mL buffer (pH 5.5, sodium acetate) in the beaker with a magnetic bar. During the entire experiment, temperature was maintained at 60 °C for Cp-CelD, while it was set up at 50 °C for Cp-Eg1. Desized round-shaped denim fabric was treated with preheated crude leaf extracts (Cp-Eg1, Cp-CelD) and commercial enzyme Novoprime 868 for 1 h. Desized denim fabric with 50 mM sodium acetate buffer served as negative control. After completion, the denim fabric was rinsed twice with deionized water followed by tap water and dried overnight. Dried denim fabric was observed for puckering/pepper-salt effect. Enzyme stability was evaluated in three aliquots (technical replicates) by measuring the release of glucose spectrophotometrically (Abs_{540}) using dinitrosalicylic acid before and after the experiment (Verma *et al.*, 2010).

Biopolishing of knitted fabric

The biopolishing of newly woven knitted fabric was carried out with chloroplast-derived endoglucanase (Cp-CelD, Cp-Eg1) and Cellusoft Supreme 22500 to evaluate removal of fuzziness and track clarity. Crude leaf extract was prepared from lyophilized biomass of Cp-CelD and Cp-Eg1 in 50 mM sodium acetate buffer (pH 5.5) as described earlier in denim experiment. Biopolishing experiment was done in a beaker in a total volume of 25 mL for 1 h. Temperature was maintained at 60 °C for Cp-CelD and 50 °C for Cp-Eg1. The required dose of the enzymatic solution and rectangular piece of knitted fabrics was added to pre-heated buffer pH 5.5. Knitted fabric with 50 mM sodium acetate buffer served as negative control. After 1-h treatment, knitted fabric was removed and rinsed twice with deionized water followed by tap water and left overnight for drying. Three aliquots of enzymes were collected before and after the experiment and assayed for endoglucanase activity. Scanning electron microscopy (FEI Quanta 250) was used to study the surface morphology of the treated and untreated knitted fabric.

Mustard oil stain removal

Industrial validation of Cp and LP-10 lipase for mustard oil stain removal was performed in 25 mL water mixed with 125 mg base detergent (Roma Laundry Detergent, Fábrica de Jabón la Corona, Mexico) and lipase enzyme (0.5% of base detergent). Equivalent units of Cp and LP-10 lipase were used in separate beakers. Water and detergent without lipase enzyme were used as control. Three aliquots of enzyme sample from each experimental setup at the start and end of the destaining experiment were collected for estimation of lipase activity using pNPB as substrate. The pH of samples in each experimental setup at the start and end of destaining experiment was measured. Destaining experiment was performed at 30 and 70 °C separately for 30 min with continuous stirring. After completion of the experiment, destained fabric was washed three times in water and dried overnight. Dried destained fabric was visualized with necked eyes and photographed to evaluate cleansing effect. The effect was again confirmed by measuring the reflectance of destained fabric by SS5100H dual beam spectrophotometer (Premier Colorscan Instruments, Navi Mumbai, India).

Mannanase enzyme assay and chocolate stain removal

Transplastomic lines expressing Cp-mannanase (PH) were grown, leaf harvested, lyophilized, ground into powder. Total soluble protein was extracted from lyophilized Cp-mannanase and WT in 50 mM sodium citrate buffer pH 5, protein estimated and assay performed as described by Agrawal *et al.*, 2011;. Temperature optima of Cp-mannanase and commercial Mannaway were compared in the range of 20–80 °C. The enzyme assays performed using three independent biological samples. Cp-mannanase and Mannaway® (Novozymes) chocolate stain removal was evaluated as described by Srivastava and Kapoor, 2014, with some modifications. Chocolate syrup (Hershey) stain prepared on fabric and kept overnight at 37 °C to stabilize stain. In destaining experiment, chocolate stained fabric was incubated in 25 mL of 50 mM sodium citrate buffer pH 5 having 125 mg of detergent and respective enzyme (0.5% of base detergent) and incubated at 30 and 70 °C separately with continuous stirring. Equivalent enzyme units of Cp-mannanase and Mannaway were tested in separate experiments. Detergent without enzyme served as negative control. Three aliquots of enzyme sample were collected at the start and end of the experiment to analyse enzyme activity. After experiment, fabric was washed with water and dried overnight. Dried destained fabric was visualized with necked eyes and photographed to evaluate cleansing effect. Cp-mannanase and Mannaway cleansing effect were compared by measuring the reflectance of destained fabric by SS5100H dual beam spectrophotometer (Premier Colorscan Instruments, India).

Statistical analysis

For temperature and pH optima studies, both chloroplast-derived and commercial enzymes were analysed using three independent biological samples. Enzyme activity assay before or/and after stain removal, biowashing and biopolishing were performed in aliquots from each beaker assay. For all experiments, mean and standard deviation (SD) values were calculated using Microsoft® Office Excel. The enzyme activity of all commercial products was performed on equivalent enzyme activities, measured under identical conditions because all commercial product labels did not provide enzyme units or weight of non-enzyme filler materials. Therefore, comparison by weight or protein concentration was not feasible.

Marker-free chloroplast vectors and selection of transplastomic lines

Supporting experimental procedures Method S1.

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Conflict of interest

The corresponding author (HD) is an inventor on several patents on expression of enzymes in plant chloroplasts, the technology founder and holds equity in PhylloZyme along with the University of Pennsylvania and investors. He also chairs the PhylloZyme Scientific Advisory Board as a consultant. All other authors do not have any financial conflict to report.

Author contributions

RS, UK, TR and KM carried out the industrial validation study of enzymes on denim, knitted fabric and stain removal. HD, RS and UK wrote the manuscript. UK and SR contributed data to Figures 1 and 2a,b. RS, TR and PS contributed data for Figure 2c,d. UK and RS, TR contributed data for Figure 3. UK and KM contributed data for Figure 4. RS and TR contributed data for Figure 5. RS and UK contributed data to Figures 6 and 7. UK and KM performed scanning electron microscopy analysis. PS and TR created the lettuce marker-free plants. HD conceived this project, designed experiments and wrote/edited this manuscript except the methods section.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Commercial tobacco leaf Lipase PhylloZyme product.
Figure S2 Commercial tobacco leaf Endoglucanase PhylloZyme product.
Methods S1 Experimental procedures.